Developmental Biology **250,** 251–262 (2002)
doi:10.1006/dbio.2002.0762

Signalling in Vertebrate Limb Development

Stefan Tümpel,[1] Juan J. Sanz-Ezquerro,[1] Alison Isaac, Maxwell C. Eblaghie, Julia Dobson, and Cheryll Tickle[2]

Division of Cell and Developmental Biology, The Wellcome Trust Biocentre, University of Dundee, Dundee DD1 5EH, United Kingdom

***Tbx3*, a T-box gene family member related to the *Drosophila* gene *optomotor blind* (*omb*) and encoding a transcription factor, is expressed in anterior and posterior stripes in developing chick limb buds. *Tbx3* haploinsufficiency has been linked with the human condition ulnar–mammary syndrome, in which predominantly posterior defects occur in the upper limb. *Omb* is expressed in *Drosophila* wing development in response to a signalling cascade involving Hedgehog and Dpp. Homologous vertebrate signals Sonic hedgehog (Shh) and bone morphogenetic protein 2 (Bmp2) are associated in chick limbs with signalling of the polarising region which controls anteroposterior pattern. Here we carried out tissue transplantations, grafted beads soaked in Shh, Bmps, and Noggin in chick limb buds, and analysed *Tbx3* expression. We also investigated *Tbx3* expression in limb buds of chicken and mouse mutants and retinoid-deficient quail in which anteroposterior patterning is abnormal. We show that *Tbx3* expression in anterior and posterior stripes is regulated differently. Posterior *Tbx3* expression is stable and depends on the signalling cascade centred on the polarising region involving Shh and Bmps, while anterior *Tbx3* expression is labile and depends on the balance between positive Bmp signals, produced anteriorly, and negative Shh signals, produced posteriorly. Our results are consistent with the idea that posterior *Tbx3* expression is involved in specifying digit pattern and thus provides an explanation for the posterior defects in human patients. Anterior *Tbx3* expression appears to be related to the width of limb bud, which determines digit number.**

Key Words: **limb development; Tbx3; Shh; Bmp; chick embryo; mouse embryo; *talpid*[3].**

INTRODUCTION

A fundamental problem in vertebrate limb development is how the pattern of structures is established across the anteroposterior axis. Anteroposterior pattern is known to be controlled by positional signalling of the polarising region, a small group of mesenchyme cells at the posterior margin of the limb bud (Saunders and Gasseling, 1968; Tickle *et al.*, 1975). Considerable progress has been made in identifying several signalling molecules associated with the polarising region, including retinoic acid, Sonic hedgehog (Shh), and Bone morphogenetic proteins (Bmps), and dissecting out their relative roles (reviewed in Sanz-Ezquerro and Tickle, 2001), but rather little is known about the target genes which are expressed in response. *Tbx3*, a member of the *T-box* (*Tbx*) gene family, which encodes transcription factors, is a good candidate for a target gene of polarising region signalling and thus to contribute to positional information.

Tbx genes belong to a family of transcription factors, which are expressed at many different stages and regions in vertebrate embryos. Of particular interest with respect to limb development are *Tbx-2*, *-3*, *-4*, and *-5*, which show a remarkable degree of evolutionary conservation from species to species and are all expressed in the limb. *Tbx4* is expressed throughout the leg bud, while *Tbx5* is expressed throughout the wing bud in chick embryos, and there is evidence that these genes are involved in specifying limb-type identity (Gibson-Brown *et al.*, 1998; Isaac *et al.*, 1998; Logan *et al.*, 1998; Ohuchi *et al.*, 1998; Rodriguez-Esteban *et al.*, 1999; Takeuchi *et al.*, 1999). *Tbx2* and *Tbx3* expression patterns in developing chick limb buds are extremely similar and consist of a series of stripes in both wing and leg buds (Gibson-Brown *et al.*, 1998; Isaac *et al.*, 1998; Logan *et al.*, 1998). For both genes, there is a posterior stripe of expression separated from a second stripe of expression at

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. Fax: +01382 345893. E-mail: c.a.tickle@dundee.ac.uk.

0012-1606/02 $35.00

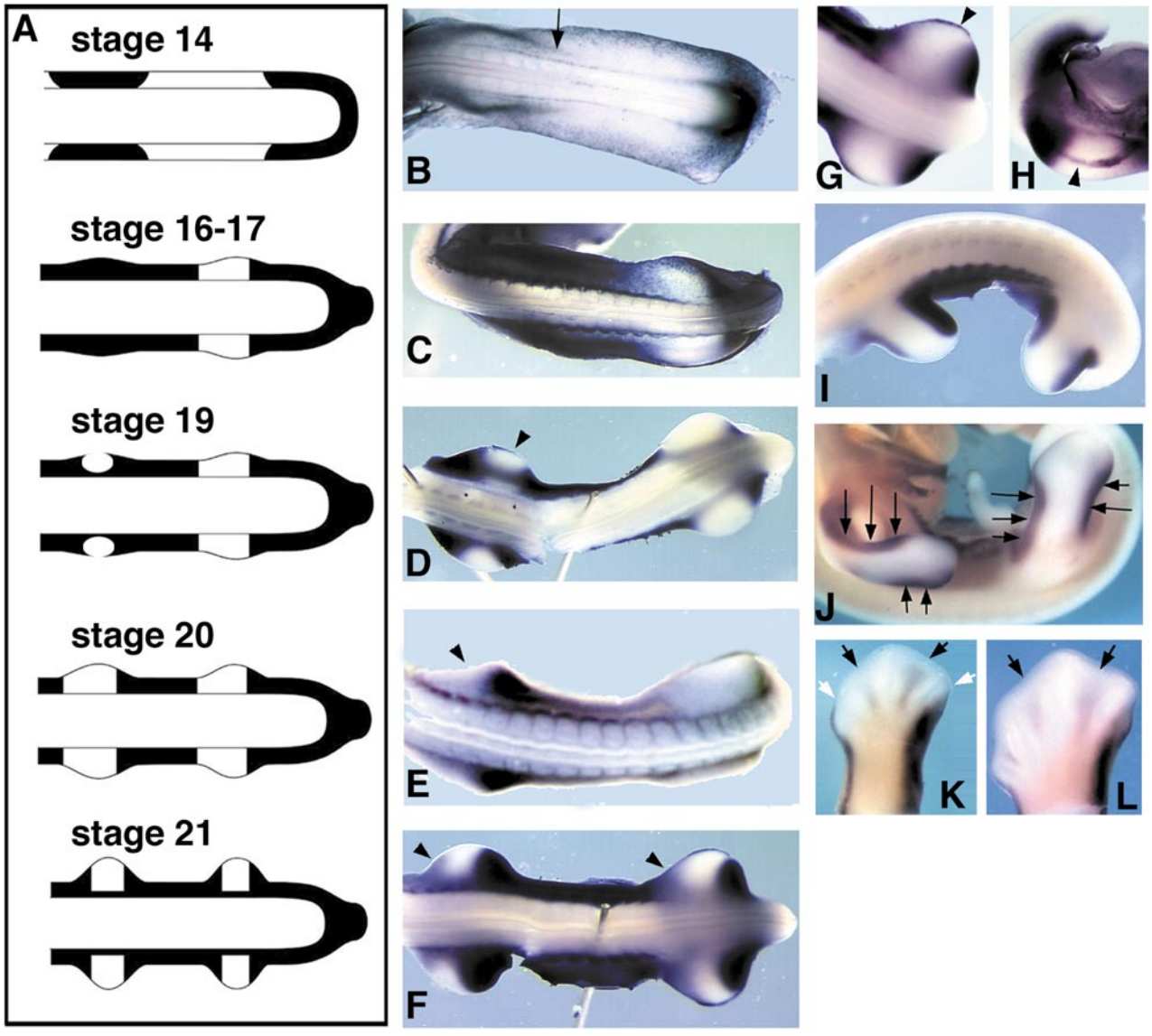

FIG. 1. *Tbx3* expression patterns in chick embryos; all dorsal views except (H). (A) Schematic representation of *Tbx3* expression patterns in lateral plate mesoderm and limb buds at different stages in chick development. Note dynamic expression in prebud and limb bud stages. Anterior to the left. (B) At stage 14 (23 somites), *Tbx3* is expressed throughout anterior lateral plate mesoderm including anterior presumptive wing (posterior limit of wing expression is opposite somite 18, arrow), but is absent from posterior presumptive wing, presumptive flank, and leg. *Tbx3* is also expressed in posterior lateral plate, tail bud, and tail fold. (C) At early stage 17, *Tbx3* is expressed throughout lateral plate mesoderm, including the whole wing-forming region and flank, and at posterior of leg-forming region. It is not expressed in anterior leg. (D) At stage 19, a distal central region where *Tbx3* is not expressed appears in the wing bud (arrowhead) and progresses anteriorly, while Tbx3 is still expressed only posteriorly in the leg bud. (E) At early stage 20 *Tbx3* is expressed in posterior wing, anterior flank, posterior leg, and tail. Expression is absent from anterior wing (arrowhead) and anterior leg buds. (F) At stage 21, *Tbx3* is expressed in characteristic striped pattern at anterior (arrowheads) and posterior of wing and leg buds and throughout flank. *Tbx3* is not expressed in the middle of the limb buds. (G) Leg buds at stage 22 showing expression anteriorly and posteriorly and in apical ectodermal ridge (arrowhead). (H) Ventral view of same limbs pointing at ridge expression (arrowhead). (I) At stage 23, anterior and posterior stripes of *Tbx3* expression are evident in both wing and leg buds. (J) At stage 26–27, *Tbx3* expression remains strong along anterior and posterior edges of wing and leg buds (arrows). (K) Stage 28 leg buds. Expression is detected in interdigital spaces (black arrows) and in anterior and posterior edges of the limb up to the distal-most part of autopod (white arrows). The posterior stripe is clearly much wider than the anterior stripe. (L) At stage 30 leg buds, expression is still present in interdigital spaces (arrows) and in edges of autopod. Posterior margin of toe 4 coincides with border of posterior *Tbx3* expression.

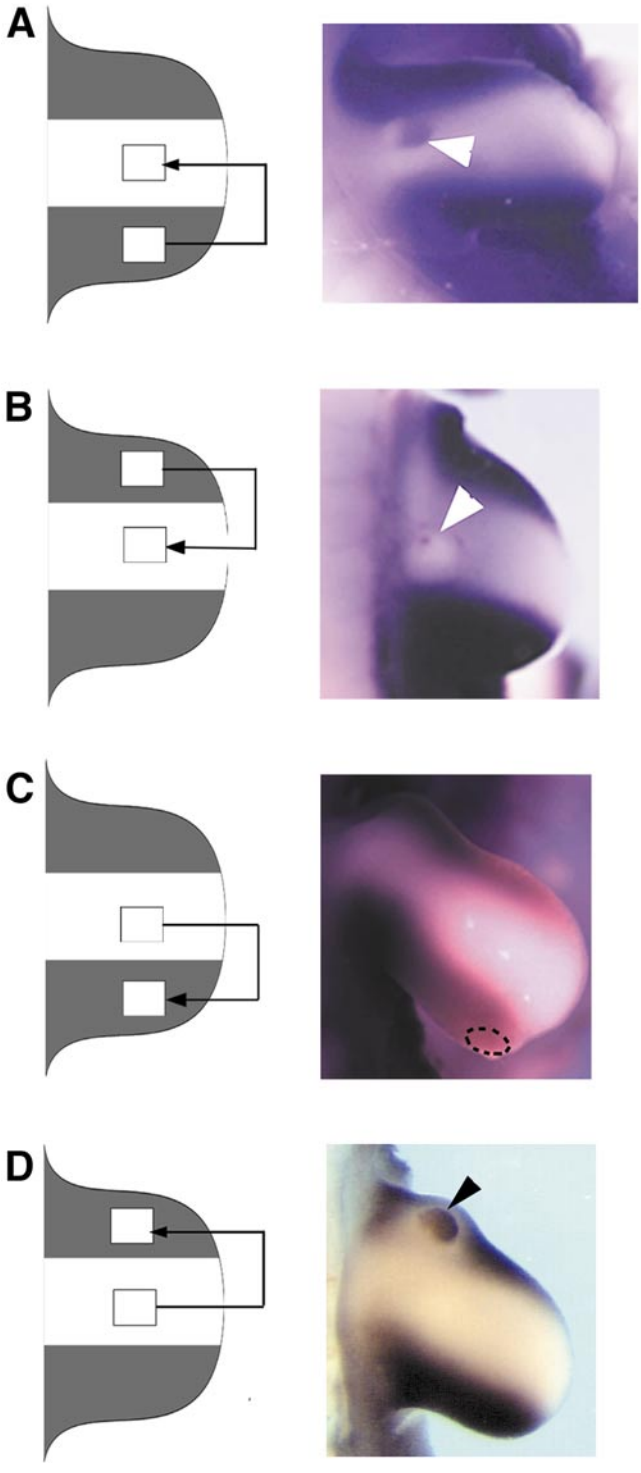

TABLE 1

Chick Limb Bud Tissue Expressing or Not Expressing *Tbx3* Grafted to Different Regions of the Limb Bud (Host Embryos Stages 20–22)

Operation	Graft St. 20–21 *Tbx3* expression in graft Number of cases (%)	Graft St. 22–23 *Tbx3* expression in graft Number of cases (%)
A. Posterior wing to middle wing	7/10 (70)	2/2 (100)
B. Anterior wing to middle wing	0/12 (0)	3/12 (25)
C. Middle wing to posterior wing	6/8 (75)	n.d.
D. Middle wing to anterior wing	3/8 (37)	5/12 (42)
E. Posterior wing to middle leg	6/10 (60)	n.d.
F. Anterior leg to middle wing	0/16 (0)	n.d.

Note. n.d., not done.

the anterior by a stripe running across the middle of the limb bud, where there is no expression.

A number of lines of evidence suggest that *Tbx3* in particular might be involved in specifying anteroposterior positional information. In human patients, haploinsufficiency of *Tbx3* leads to ulnar–mammary syndrome in which posterior structures are missing in the upper limb (Bamshad *et al.*, 1999; Bamshad, 1997). Furthermore, *omb*, the *Drosophila* gene most closely related to *Tbx3* and *Tbx2*, is known to be a target of a homologous signalling cascade in *Drosophila* wing patterning to that which operates in vertebrate limb patterning. In *Drosophila* wing development, *omb* expression is established in response to signalling by Dpp, which is induced by Hh (Grimm and Pflugfelder, 1996; Lecuit *et al.*, 1996; Nellen *et al.*, 1996). In chick wing development, Shh expressed in the polarising region induces expression of *Bmp2*, a vertebrate homologue of *dpp*, and we recently suggested that Shh and Bmps cooperate in patterning the anteroposterior axis of the limb (Drossopoulou *et al.*, 2000; Yang *et al.*, 1997). Shh expression in vertebrate limbs appears to be induced in response to retinoic acid (Laufer *et al.*, 1994; Niswander *et al.*, 1994; Stratford *et al.*, 1999). It has been reported that *Tbx2*

FIG. 2. *Tbx3* expression in grafts of tissue moved along anteroposterior axis of wing bud. A schematic representation of graft performed is on the left in each case; an example of the result is on right. Mesenchymal grafts were taken from stage 20–21 donor wing buds and were implanted into stage 20 host wing buds and embryos collected 24 h later. (A) Tissue grafted from posterior *Tbx3* stripe to middle nonexpressing stripe. Graft expresses *Tbx3* (arrowhead). (B) Tissue grafted from anterior *Tbx3* stripe to middle nonexpressing stripe. Grafted tissue does not express *Tbx3* (arrowhead). (C) Tissue grafted from middle nonexpressing region to posterior *Tbx3* stripe. Grafted tissue expresses *Tbx3* (dotted circle marks position of graft identified by DiI label prior to *in situ* hybridisation). (D) Tissue was grafted from middle nonexpressing region to anterior *Tbx3* stripe. *Tbx3* expression is induced in graft tissue (arrowhead).

expression can be induced in chick limbs by Shh (Gibson-Brown *et al.*, 1998).

Here we explore whether *Tbx3* expression in vertebrate limb buds is controlled by polarising region signalling by carrying out tissue transplantations and grafting beads soaked in signalling molecules in chick limb buds and by monitoring *Tbx3* expression in limb buds of mutants (chicken *talpid*^{3} and mouse *Xt/Xt* and *Shh* null) and retinoid-deficient quail embryos, in all of which the polarising region cell–cell signalling cascade is abnormal.

MATERIALS AND METHODS

Embryos

Fertilised chicken eggs (White Leghorn, Hisex breed from Needle farm, UK) were incubated at 38°C until the required stage. Fertilised *talpid*^{3} eggs were obtained from Dave Burt and Dave Morrice (Roslin Institute). Stage 20/21 retinoid-deficient quail embryos (Stratford *et al.*, 1999) were obtained from Malcom Maden (King's College London). Homozygous *Xt/Xt* (10.5 days; Zuniga and Zeller, 1999) and Shh$^{-/-}$ (11 days; Zuniga *et al.*, 1999) mouse embryos and wt or heterozygous littermates were provided by Rolf Zeller (Utrecht University).

Whole-Mount in Situ Hybridisation

Whole-mount *in situ* hybridisations of chick, quail, and mouse embryos were carried out by using standard protocols as previously described (Nieto *et al.*, 1996; Wilkinson and Nieto, 1993). DIG-labelled RNA probes were made by using standard protocols. Chicken Tbx3 was a gift from Juan-Carlos Izpisúa-Belmonte (Salk Institute; Isaac *et al.*, 1998); chicken Bmp4 has been described in Francis *et al.* (1994); for mouse Tbx3, a 506-nucleotide cDNA fragment was obtained by RT-PCR from 11.5 days embryo total RNA and cloned in pGEM-T. Primers used were: forward, 5′-GAGATGGTCATCACGAAGTC-3′; reverse, 5′-CTGCAATGCCCAATGTCTCG-3′. The sequence obtained has been deposited in GenBank (Accession No. AF429310).

Tissue Grafts

Grafts of tissue from wing to wing and wing to leg and polarising region grafts were carried out by using an adaptation of the method described by Saunders (1957). Donor grafts from Hamburger and Hamilton stage 20–21 or 22–23 (Hamburger and Hamilton, 1951) were incubated in 2.5% trypsin on ice for 10 min and transferred to growth medium (MEM; 25 mM Hepes plus 10% foetal calf serum, 1% penicillin–streptomycin, and 2 mM glutamine), and the ectoderm was removed. In some cases, prior to implantation, grafts were labelled for 30 min at 37°C in a 9-μg/ml DiI solution (made in growth medium from a 3-mg/ml DiI stock in dimethyl formamide) to distinguish unequivocally grafted from host tissue later on. Grafts were inserted into the mesenchyme of host limb buds (stages 20–22) at different positions across the anteroposterior axis or directly beneath the apical ridge at the anterior or posterior of the limb bud. Operated embryos were incubated for 24–48 h, then fixed in 4% PFA in PBS and processed for *in situ* hybridisation.

Bead Implantation

Affigel Blue CM beads (Bio-Rad Laboratories Ltd) were used for Shh and Noggin; heparin acrylic beads (Sigma H-5263) were used for Bmp2. Beads were soaked in 4 mg/ml Shh (recombinant mouse amino-terminal peptide from R&D Systems), 0.1 mg/ml Bmp2 (recombinant human Bmp2; a gift from the Genetics Institute), or 1 mg/ml Noggin (recombinant mouse Noggin/Fc chimera, from R&D Systems) for 1 h at room temperature. Beads were inserted at different positions into the mesenchyme of stage 18–22 chick embryos or in the third interdigital space of stage 27–28 embryos. Eggs were incubated further until they reached the desired stage and fixed in 4% PFA–PBS, and whole-mount *in situ* hybridisation was carried out as above.

RESULTS

Tbx3 Expression in Chick Embryos

In chick embryos, *Tbx3* is expressed in a clear striking striped pattern in both wing and leg bud mesoderm, with an anterior and a posterior stripe of cells expressing *Tbx3* separated by a stripe of cells that do not express *Tbx3*. The two stripes of *Tbx3* expression appear by a highly dynamic route that is different in wing and leg (see scheme in Fig. 1A). At stage 14, *Tbx3* is expressed anteriorly in presumptive wing region but not posteriorly nor in flank, but is strongly expressed at the posterior end of the embryo (Fig. 1B). By stage 16–17, *Tbx3* is expressed throughout presumptive wing region, flank, and in posterior, but not anterior of presumptive leg (Fig. 1C). By stage 19, a central distal region where *Tbx3* is not expressed begins to appear in the wing bud, while in the leg bud, *Tbx3* is still expressed only posteriorly (Fig. 1D; Isaac *et al.*, 1998). In slightly later embryos, *Tbx3* expression is further reduced in the anterior wing just leaving a posterior stripe as in the leg (Fig. 1E; Gibson-Brown *et al.*, 1998). By late stage 20–21, *Tbx3* expression now appears anteriorly in both wing and leg buds and the striped pattern is established (Fig. 1F; Gibson-Brown *et al.*, 1998; Isaac *et al.*, 1998; Logan *et al.*, 1998). At this stage, *Tbx3* is also expressed in the apical ectodermal ridge of the leg buds (Figs. 1G and 1H). *Tbx3* is expressed along both anterior and posterior edges of the limb buds during further outgrowth (see for example stage 23 in Fig. 1I) until stage 26 (Fig. 1J). At stages 27–30, *Tbx3* is still expressed along anterior and posterior edges of the limbs, including the hand and foot plates right up to the distalmost part of the autopods with the posterior stripe being wider (Figs. 1K and 1L). By stage 28 (Fig. 1K), interdigital expression is also established, which persists during stages before the onset of interdigital cell death (see stage 30, Fig. 1L). At stage 32, interdigital expression becomes restricted to the edges of digital rays.

Tbx3 Expression in Grafts

In order to investigate how the pattern of *Tbx3* expression is controlled, we grafted chick limb bud tissue from regions

which express *Tbx3* to regions that do not express *Tbx3* and vice versa (Table 1; Fig. 2).

When tissue from stage 20–21 embryos was taken from the posterior *Tbx3* stripe of wing buds and grafted to the middle nonexpressing stripe, *Tbx3* expression was maintained in most cases (70%) (Table 1A; Fig. 2A). In contrast, when tissue was taken from the anterior *Tbx3* stripe and grafted to the middle, *Tbx3* expression was lost in all cases (Table 1B; Fig. 2B). In addition, when anterior tissue taken from an early stage 20 host wing bud, in which *Tbx3* expression would not have appeared yet, was grafted to the middle of the bud, *Tbx3* expression does not come on. Similar grafting experiments were carried out by using tissue derived from stage 22–23 wing buds. When anterior tissue already expressing *Tbx3* strongly at this stage (as checked by fixing some host embryos immediately following the operation) was placed in the middle of the bud, only 25% of grafts still expressed *Tbx3* 20 h later, and this expression was weak in two of the cases. In contrast, as with tissue from younger limb buds, when posterior tissue was grafted to the middle, expression was retained (Table 1A). Thus, *Tbx3* expression in posterior limb bud cells seems much more stable than *Tbx3* expression in anterior cells.

When tissue from the middle nonexpressing region of the wing was grafted to the posterior *Tbx3* stripe of the wing, *Tbx3* expression was induced in most cases (75%; Table 1C, Fig. 2C), while when this tissue was grafted to the anterior *Tbx3* stripe, *Tbx3* expression can also be induced, but less efficiently (37% for stage 20–21 grafts, 42% for stage 22–23 grafts; Table 1D, Fig. 2D). Thus, *Tbx3* expression was switched on more efficiently when cells from the nonexpressing stripe are grafted posteriorly compared with anteriorly.

Tissue was also grafted between wing and leg buds to check whether the same rules apply in both wing and leg. Tissue from the posterior *Tbx3*-expressing stripe in wing was grafted to the middle nonexpressing region of leg, and leg tissue was grafted from anterior *Tbx3*-expressing stripe to the middle, nonexpressing region of wing (Table 1, E and F). The grafts behaved in the same way as if they had been transplanted within the confines of the wing bud.

Regulation of Tbx3 Expression by Shh and Bmps

It has previously been shown that *Tbx2*, which is highly related to *Tbx3*, can be switched on by Shh (Gibson-Brown *et al.*, 1998), and therefore, one possibility is that Shh regulates *Tbx3* expression in the posterior stripe. To test whether Shh can switch on *Tbx3* expression, beads soaked in Shh were implanted at various positions in stage 18–22 wing buds, and the effects on *Tbx3* expression were monitored 16–24 h later. Implantation of Shh beads to the middle of the limb buds or slightly posteriorly induced local *Tbx3* expression resulting in either expansion of the posterior stripe or a patch of ectopic *Tbx3* expression (n = 24/26 and n = 11/13, respectively; Figs. 3A and 3B, arrowheads). The same result was obtained in legs (n = 4/6). The extent and intensity of ectopic expression was more marked when beads were implanted in earlier embryos (stage 18–19) and when analysed at shorter times after bead implantation (compare Fig. 3A, stage 19 at 18 h, with Fig. 3C, stage 20 at 24 h). In addition, in some cases, anterior *Tbx3* expression was noticeably reduced (Figs. 3A and 3B, arrows). Indeed, when Shh beads were placed directly in the anterior of limb buds, marked reduction of anterior *Tbx3* expression was observed at 16–22 h later in both wing (n = 10/10) and leg (n = 2/2) buds (Fig. 3D). Sometimes, a distal patch of *Tbx3* expression was present (see below Fig. 3F, white arrowhead). Thus, Shh appears to positively regulate *Tbx3* expression in the middle and posterior of the limb bud, while it negatively regulates *Tbx3* expression in the anterior.

When Shh is applied to the anterior of a limb bud, this respecifies anterior cells to form posterior structures. Thus, it was surprising to find reduction of *Tbx3* expression anteriorly, since posterior tissue should express *Tbx3*. Therefore, we followed *Tbx3* expression in Shh-treated stage 20 wing buds for a longer period and also in limb buds to which a polarising region had been placed anteriorly. Some of these embryos were left to develop until 10 days and full mirror image duplications or extra digit 3's were observed, confirming reprogramming of anterior tissue to form posterior structures. In these experiments, at 22 h, as seen before, expression of *Tbx3* was absent distally (n = 4/4), but by 48 h, *Tbx3* expression had returned along the anterior margin of the bud (n = 4/4; Fig. 3E). The same result was obtained after polarising region grafts; anterior *Tbx3* expression is reduced distally in the host at 24 h (n = 13/15; Fig. 3F, arrow), but *Tbx3* expression is reestablished along the anterior margin by 48 h (n = 8/10; Fig. 3G, arrow). At both time points, the graft itself continues to express *Tbx3* (Figs. 3E and 3F, black arrowheads); grafted tissue was recognised by DiI labelling. These results indicate that the reprogramming of anterior tissue occurs in two steps: first, anterior expression of *Tbx3* is inhibited, and then, *Tbx3* expression reappears in connection with induction of extra digits with posterior identity.

Analysis of *Tbx2* and *Tbx3* expression in other regions of embryos has implicated Bmps as being the regulatory signals (Yamada *et al.*, 2000). Since Bmp2 is downstream of Shh in chick limb buds, we explored the possibility that the Shh effects are mediated by Bmp2. Beads soaked in Bmp2 were placed at various positions in stage 19–21 wing buds, and *Tbx3* expression was analysed. In wing buds of all embryos collected at 8 h (n = 16/16 in which the bead had been placed anteriorly, 22/22 centrally, 8/8 posteriorly), there was clear ectopic expression in the central nonexpressing stripe and/or expansion of either anterior (Fig. 4A) or posterior stripe of *Tbx3* expression (Fig. 4B). It was even possible to induce prematurely anterior *Tbx3* expression by applying Bmp2 for 8 h. However, when embryos were analysed 16–18 h after Bmp2 bead implantation, ectopic *Tbx3* expression in the central region of buds was weaker (n = 10/32; Figs. 4C and 4D, arrowheads) or absent, but

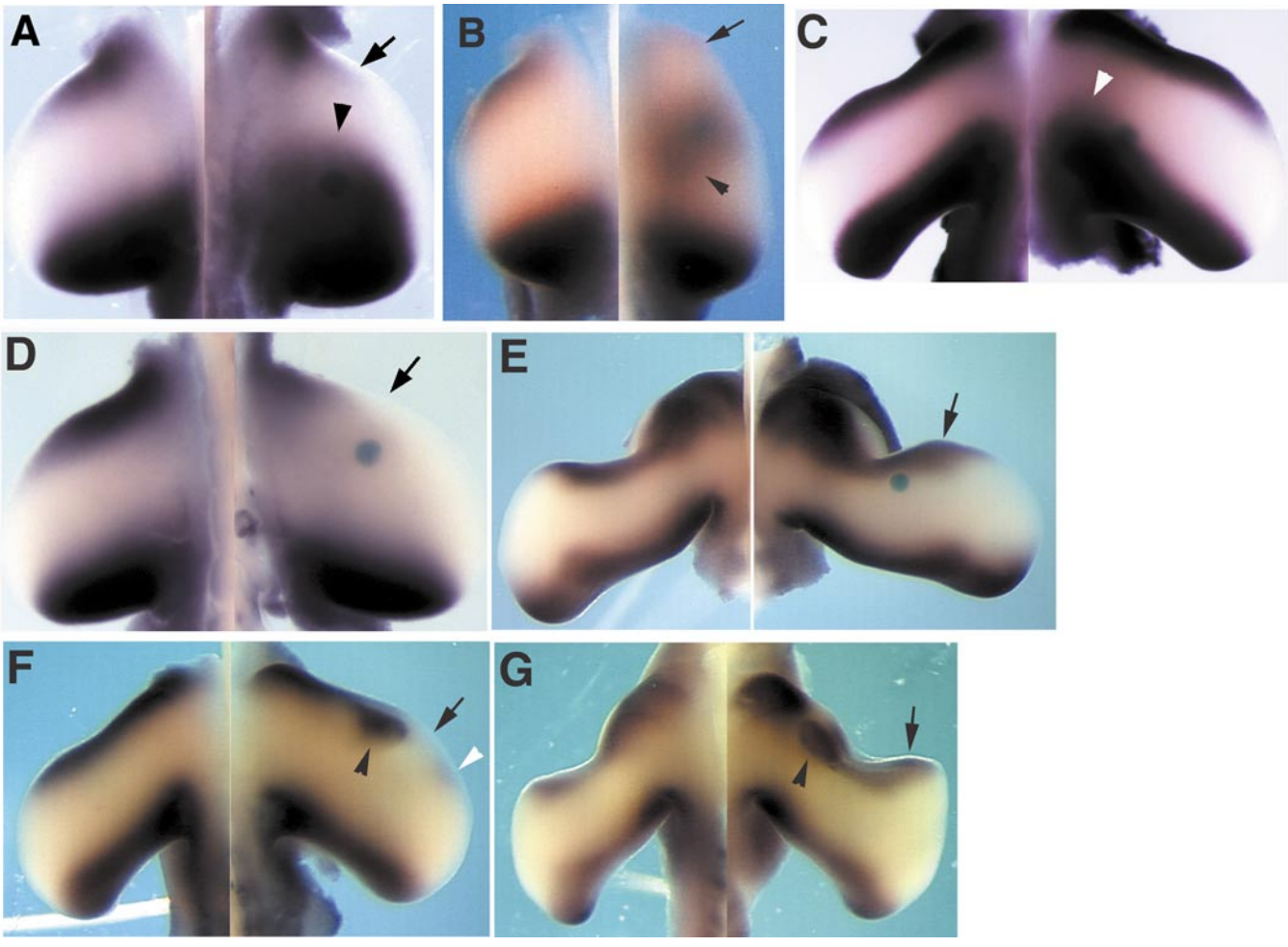

FIG. 3. Effects of Shh application on *Tbx3* expression. (A) Shh bead placed in middle of a stage 19 wing bud and expression of *Tbx3* analysed 18 h later. Note expansion of posterior stripe into middle of bud (arrowhead) and reduction of anterior stripe (arrow). (B) Shh bead placed at the apex of a stage 19 wing bud and expression of *Tbx3* analysed 16 h later. Note diffuse ectopic *Tbx3* expression in middle of bud (arrowhead) and reduction of anterior stripe (arrow). (C) Shh bead placed in middle of a stage 20 wing bud and expression of *Tbx3* analysed 24 h later. Note low level residual expansion of *Tbx3* domain proximally (arrowhead). (D) Shh bead placed in anterior of a stage 19–20 wing bud and expression of *Tbx3* analysed 18 h later. Note reduction of anterior *Tbx3* expression stripe (arrow). (E) Forty-eight hours after an anterior Shh bead was implanted, expression of *Tbx3* is restored along the anterior margin of the bud (arrow). (F) Twenty-four hours after a polarising region graft, expression of *Tbx3* is reduced anteriorly in the host (arrow). Note a small patch of distal expression (white arrowhead). (G) Expression of *Tbx3* is reestablished in the host 48 h after a polarising region graft (arrow). Black arrowheads in (F) and (G) point to the graft (identified by DiI labelling prior to *in situ* hybridisation), which itself expresses *Tbx3*.

there was no cell death as detected by Nile Blue Sulphate. In contrast, with Bmp2 beads placed close to the ridge, there is a local reduction in bud width accompanied by reduction in the normal expression domains (n = 15/15; Fig. 4C, arrow), due to induction of cell death in those areas as checked by Nile Blue Sulphate staining. These results indicate that Bmp2 can induce *Tbx3* expression rapidly throughout the limb bud, but this effect appears to be transient in the central stripe, where *Tbx3* is normally not expressed, and ectopic expression is soon switched off again.

To analyse further the involvement of Bmps in the regulation of *Tbx3* expression, we made use of the Bmp inhibitor Noggin (Zimmerman *et al.*, 1996). Beads soaked in Noggin were placed at various positions in wing buds at stage 19–22. With Noggin beads placed anteriorly, there was a dramatic reduction in *Tbx3* expression in the anterior stripe 16–22 h later (n = 7/8 at stage 19–20, n = 1/1 at stage 22; Fig. 4E), although again, as with Shh beads, a distal area of *Tbx3* expression was present sometimes. To demonstrate the specificity of action of Noggin on Bmp activity, a Bmp2 bead was placed proximal to the Noggin bead in the anterior part of the limb in two cases. In this experiment, expression

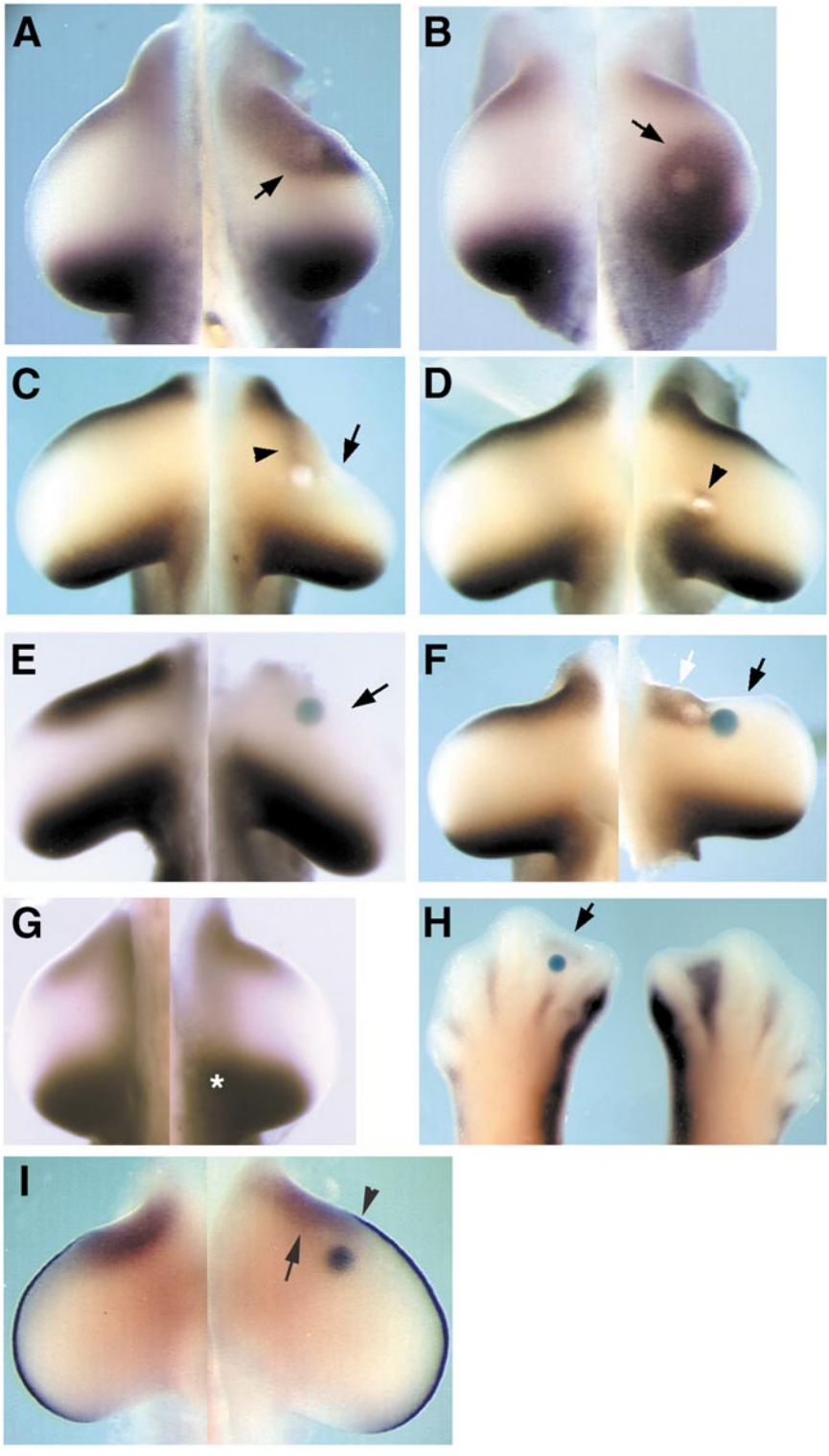

FIG. 4. Effects of Bmp2 and Noggin on *Tbx3* expression. (A) Bmp2 bead placed in anterior of a stage 20 wing bud. *Tbx3* expression analysed 8 h later. Note expansion of anterior stripe close to the bead into the middle of the bud (arrow). (B) Bmp2 bead placed in middle of stage 20 wing bud, in the *Tbx3* nonexpressing area. Eight hours later, expansion of the posterior stripe and ectopic expression in the middle (arrow) can be observed. (C) Bmp2 bead was placed in anterior of stage 20 wing bud and *Tbx3* expression analysed 18 h later. Note residual expression of the expanded anterior stripe fading off (arrowhead, compare with A) and decrease of limb width and accompanying loss of

of *Tbx3* around the Bmp2 bead was strong, but disappeared close to the Noggin bead (Fig. 4F). This confirms that Noggin blocked *Tbx3* expression in the anterior stripe by interfering with Bmp signalling. In contrast to these effects of Noggin on anterior *Tbx3* expression, there was no apparent effect on posterior *Tbx3* at 16–22 h (n = 23/23; Fig. 4G). Furthermore, Noggin beads were not able to block expansion of the posterior stripe of *Tbx3* expression when simultaneously implanted with Shh beads at the apex of the nonexpressing stripe (n = 6/6). When Noggin beads were placed in the third interdigit of leg buds at stage 27, the same effects were observed as with the anterior stripe in the limb bud, and expression of *Tbx3* was reduced 24 h later (Fig. 4H).

The results described above show that *Tbx3* expression in the anterior stripe is dependent on continuous Bmp signalling, but *Bmp2* is not expressed in anterior mesenchyme. However, transcripts of a gene encoding another Bmp, *Bmp4*, are found at high levels in this region of chick limb buds (Francis *et al.*, 1994) and could regulate *Tbx3* expression anteriorly. Since we had found that the anterior stripe of *Tbx3* expression was inhibited by Shh signalling, we therefore analysed expression of *Bmp4* after anterior application of Shh beads. When Shh beads were placed anteriorly at stage 20 and *Bmp4* expression was analysed 16 h later, a reduction in its mesenchymal expression domain was clearly observed (n = 14/15; Fig. 4I, arrow). Thus, Shh appears to reduce *Tbx3* expression in the anterior stripe by inhibiting expression of *Bmp4* in the mesenchyme. Indeed, when Bmps are applied together with Shh, *Tbx3* expression is rescued. In contrast to reduction of *Bmp4* expression in the mesenchyme, *Bmp4* expression was maintained in the extended apical ectodermal ridge present after Shh bead application (Fig. 4I, arrowhead).

Finally, we examined *Tbx3* expression patterns in limb buds of various embryos with abnormal Shh signalling: *talpid*^{3} mutant chicken embryos, retinoid-deficient quail embryos, and in mouse mutants, extra toes (*Xt/Xt*) and *Shh* null (*Shh*$^{-/-}$) embryos. In the *talpid*^{3} mutant, in which it has been suggested that Shh protein diffuses widely throughout the limb bud (Lewis *et al.*, 1999) and *Bmp2* and *Bmp4* are expressed uniformly across early limb buds (Francis-West *et al.*, 1995), *Tbx3* was expressed in the middle region of both wing and leg buds, normally free of *Tbx3* expression at these stages (Fig. 5A, arrowheads). These results are consistent with Bmp signalling regulating *Tbx3* expression. In later stage *talpid*^{3} embryos (stage 24 and 26–27), there is strong expression along the posterior border of the limbs, excluding the distal-most area which will form digits, while anteriorly there is a proximal patch of expression (Fig. 5B, wings, and 5C, legs). Although both anterior and posterior *Tbx3* expression is confined more proximally in the mutant, anterior expression is much more restricted than posterior expression (compare Figs. 5B and 5C; arrowheads with Fig. 1J). In between these patches of expression, there appears to be a region of very low expression with a clear rim separating this from the distal region of the limb bud where *Tbx3* is not expressed (Fig. 5D). In *Xt/Xt* homozygous mouse embryos, where there is ectopic anterior expression of *Shh* due to a mutation in the gene encoding the transcription factor *Gli3* (Masuya *et al.*, 1995), *Tbx3* expression was absent from the anterior stripe (compare normal mouse limb bud in Fig. 5E with *Xt/Xt* limb bud in Fig. 5F). These patterns of expression should be contrasted with those seen in limb buds where the Shh signalling pathway is absent (retinoid-deficient quail and *Shh* knock-out mice). In retinoid-deficient quail embryos, *Shh* and *Bmp2* expression is absent in leg buds (Stratford *et al.*, 1999) and posterior *Tbx3* expression is missing. Moreover, in the absence of Shh, anterior *Tbx3* expression in the leg buds is expanded (compare normal quail in Fig. 5G, arrowhead with RA-deficient quail in Fig. 5H, arrows). It should be noted that, in the wing buds of these retinoid-deficient quail embryos, *Shh* and *Bmp2* expression is still present and no reduction of *Tbx3* expression is observed (Fig. 5H, arrowheads). Similarly, in Shh null embryos, the posterior stripe of *Tbx3* expression is absent, but the anterior stripe is expanded posteriorly (compare normal limb in Fig. 5I with mutant limb in Fig. 5J). All these data confirm a positive role for Shh signalling in the establishment and/or maintenance of posterior *Tbx3* expression and a negative role in anterior expression.

DISCUSSION

We have shown that *Tbx3* expression in the posterior and anterior of vertebrate limb buds is controlled via different

anterior normal expression (arrow), due to cell death. (D) Bmp2 bead placed in middle of stage 20 wing bud and *Tbx3* expression analysed 18 h later. Note residual ectopic expression in middle of bud (arrowhead). (E) Noggin bead placed anteriorly in stage 22 wing bud. Sixteen hours later, expression of *Tbx3* in the anterior stripe is absent (arrow). (F) Two beads were placed in anterior of stage 20 wing bud: a proximal Bmp2 bead (white) and a more distal Noggin bead (blue). Eighteen hours after bead implantation, *Tbx3* expression is present around Bmp2 bead (white arrow) but has disappeared from next to the Noggin bead (black arrow). (G) Noggin bead placed in posterior of stage 19 wing bud. Eighteen hours later, expression of *Tbx3* is not affected (asterisk marks position of bead). (H) Noggin bead placed in third interdigital space of a stage 27 leg bud. Twenty-four hours later, *Tbx3* expression in the interdigital region is reduced (arrow, compare with contralateral unoperated leg bud). (I) Shh bead placed in anterior of stage 20 wing bud. Sixteen hours later, expression of *Bmp4* was analysed. Note reduction of anterior expression in the mesenchyme (arrow, compare with control limb). In contrast, *Bmp4* expression is maintained in the extended apical ectodermal ridge (arrowhead).

mechanisms (Fig. 6). Posterior *Tbx3* expression is very stable and depends on the polarising region signalling cascade; in contrast, anterior *Tbx3* expression is negatively regulated by Shh and is dependent on continuous signalling by anteriorly produced Bmps.

Analysis of retinoid-deficient quail leg buds and Shh null mutant mice show that posterior *Tbx3* expression in vertebrate limbs is dependent on polarising region signalling. Furthermore, when chick limbs are experimentally reduplicated by applying Shh or grafting a polarising region anteriorly, this is accompanied by the establishment of a stable stripe of *Tbx3* expression in the part of the limb that will form the new posterior structures. These findings suggest a parallel with the control of expression of *omb*, the *Drosophila* homologue of *Tbx3*, in *Drosophila* wing development. Here, Dpp induced by Hedgehog acts as a long-range graded signal to activate target genes, including *omb* (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). In chick wing, Shh produced by the polarising region induces Bmp2 (a Dpp homologue), and we have suggested that Bmps act as positional signals (Drossopoulou *et al.*, 2000). Therefore, the prediction would be that posterior *Tbx3* expression would be regulated by Bmps. Our results suggest, however, that it may not be that simple. Although Bmp2 can expand posterior *Tbx3* expression as expected, Shh can also expand posterior expression, acting apparently independently of Bmp signalling since coimplantation of a Noggin bead with a Shh bead cannot block this effect. Indeed, in the adult abdominal segments of *Drosophila*, *omb* expression is regulated by *Hh* and does not involve *Dpp* (Kopp *et al.*, 1997). Thus, in vertebrate limbs, both Shh and Bmp2 may act either at different times or together to establish the posterior domain of *Tbx3* expression (Fig. 6).

Our grafting experiments and experiments with Noggin show that posterior *Tbx3* expression is very stable and continuous Bmp signalling is not required. Expression of another member of the *Tbx* gene family, *Brachyury*, has been shown, in *Xenopus*, to be regulated by an autoregulatory loop involving Fgf signalling (Schulte-Merker and Smith, 1995), but there is no evidence that Fgf signalling is involved in maintaining posterior *Tbx3* expression (Gibson-Brown *et al.*, 1998; and data not shown). Furthermore, *Tbx3*, unlike *Brachyury*, is thought to encode a repressor of transcription rather than an activator (He *et al.*, 1999) and thus might operate via repressing a repressor. Indeed, the fact that ectopic *Tbx3* induced in the middle of the limb in response to Shh and to Bmp2 is transient suggests that the extent of posterior expression could be regulated by inhibitory factors found in the middle of the limb bud. For example, one could imagine that a gene that inhibits *Tbx3* expression could be activated in response to polarising region signalling but at a lower threshold level. This type of mechanism could explain our finding that *Tbx3* expression is widespread in early *talpid3* limb buds in which we have suggested that there is widespread diffusion of Shh. Another possible mechanism for restricting posterior *Tbx3* expression is that other factors present only in the posterior region of the bud are required. In either case, the regulatory mechanisms must operate rather precisely to generate the very sharp boundary to the posterior *Tbx3* expression domain.

The results of applying Bmps and Noggin to chick limb buds show that anterior *Tbx3* expression is clearly induced and maintained by Bmp signalling. Bmp4, which is expressed anteriorly, could be involved. An unexpected finding is that anterior *Tbx3* expression is negatively regulated by Shh. Application of Shh beads abolishes anterior *Tbx3* expression in chick limb buds, and anterior *Tbx3* expression is also absent in Xt/Xt mutant mice in which *Shh* is ectopically expressed anteriorly. However, it is possible that another mechanism acting upstream of ectopic *Shh* could account for this lack of expression in Xt/Xt mutant. The results of further experiments in chick limb buds suggest that this inhibitory effect of Shh on anterior *Tbx3* expression is based on interference with Bmp signalling. Thus, application of Bmp together with Shh leads to rescue of anterior *Tbx3* expression. In addition, we show that Shh reduces the extent of the anterior *Bmp4* expression domain.

Analysis of limb buds in which Shh signalling is defective points to a role for Shh in limiting the width of the anterior stripe in normal limb buds. In leg buds of retinoid-deficient quail and in limb buds of Shh null mice, the anterior *Tbx3* domain is extended. One possibility is that, in the normal limb, Shh itself acts long range to restrict *Bmp4* expression anteriorly, and recent work suggests that Shh may indeed diffuse widely within the limb bud (Gritli-Linde *et al.*, 2001; Lewis *et al.*, 2001; Zeng *et al.*, 2001). Thus, in the developing vertebrate limb bud, two opposing signals, Bmp4 emanating from the anterior and Shh from the posterior, regulate the extent of anterior *Tbx3* expression. This is reminiscent of neural tube patterning, where it is well established that Bmp4 produced dorsally opposes the effects of Shh produced ventrally (Liem *et al.*, 1995). It is interesting that Shh has also been reported to inhibit the expression of other anteriorly expressed genes, such as *Alx4* and *Gli3* (Takahashi *et al.*, 1998). Furthermore, in chick limb buds in which the apical ridge was removed posteriorly and in which Shh signalling would be expected to be impaired, there is expansion of the *Alx-4* domain posteriorly (see Fig. 4 in Takahashi *et al.*, 1998).

The finding that *Tbx3* expression is regulated by Bmp signalling in vertebrate limb buds seems to reflect a general and widely used molecular mechanism for regulating *Tbx* gene expression. For example, it has been reported that Bmp2 regulates *Tbx2* and*Tbx3* expression in the developing heart (Yamada *et al.*, 2000). Our experiments with Noggin also show that *Tbx3* expression in interdigital regions in later limb buds is regulated by Bmps. In addition, there is evidence that Bmp signalling regulates expression of *Tbx4* and *Tbx5*, genes expressed specifically in wings and legs and involved in specifying limb identity (Rodriguez-Esteban *et al.*, 1999). Expression of another *Tbx* family member, *Tbx1*, has recently been shown to be regulated by Shh during

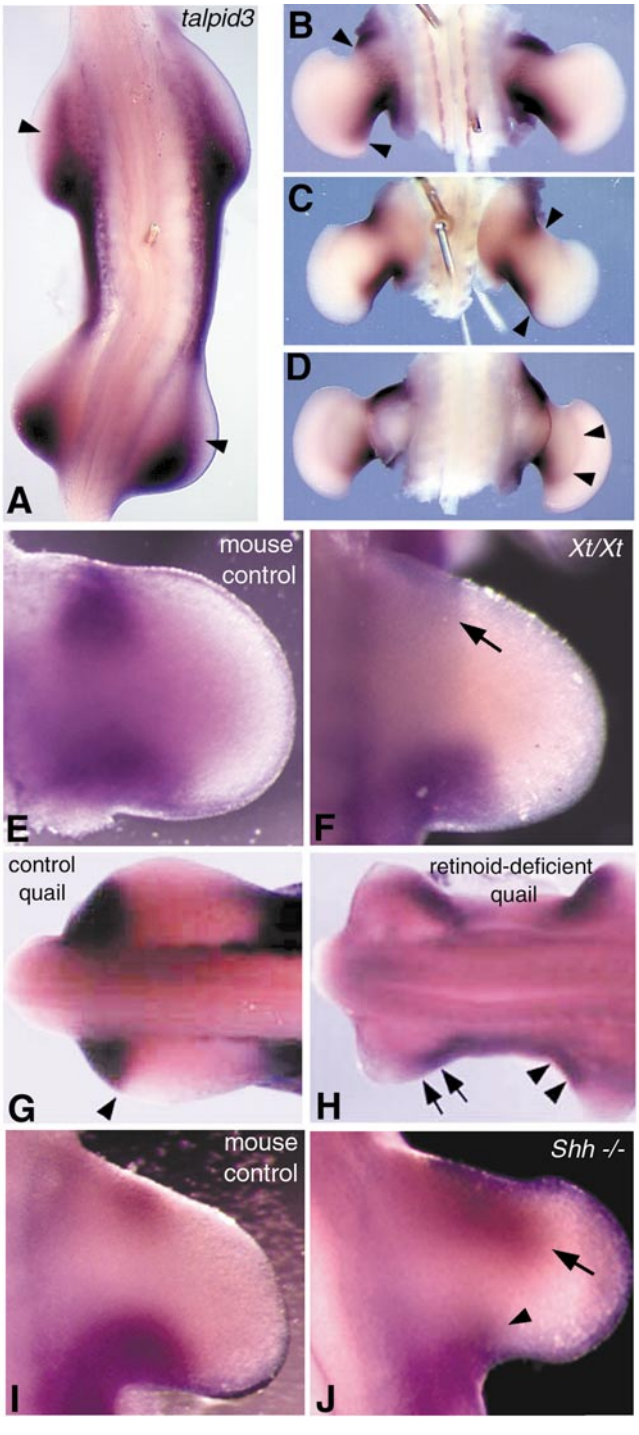

FIG. 5. Expression of *Tbx3* in limb buds of mutants and retinoid deficient quail in which Shh signalling is abnormal. (A) Expression of *Tbx3* in the chicken *talpid3* mutant at stage 20. Note widespread *Tbx3* transcripts in wing and leg buds, including the middle region (arrowheads). Expression is much weaker from distal areas. (B–D) Expression of *Tbx3* in limbs of stage 26 *talpid* embryos. (B) A dorsal view of wings. (C) A dorsal view of legs. (D) A ventral view of wings. *Tbx3* is expressed both posteriorly and anteriorly. Both anterior and posterior domains are found more proximally than in normal embryos, but the anterior domain is much more restricted than the posterior domain (arrowheads in B and C indicate distal limit of expression anteriorly and posteriorly; compare with Fig. 1J). Note in (D) (arrowheads), the rim of a region of weak expression which extends between the anterior and posterior expression domains. (E) E10.5 mouse forelimb showing anterior and posterior areas of *Tbx3* expression. (F) Forelimb of a littermate *Xt/Xt* mutant embryo. Note absence of *Tbx3* expression at the anterior (arrow). (G, H) *Tbx3* expression in normal (G) and retinoid-deficient (H) quail embryos. Note presence of the posterior stripe of expression in leg buds of normal quail (arrowhead in G) but absence in RA-deficient quail leg buds. Note also that the anterior stripe of expression in leg buds of RA-deficient embryos (arrows in H) is expanded. Posterior stripe of *Tbx3* expression is present in RA-deficient quail wing buds (arrowheads in H). (I) Expression of *Tbx3* in a E11 mouse forelimb. (J) Expression of *Tbx3* in a forelimb of a littermate $Shh^{-/-}$ null mouse embryo. Note absence of posterior expression domain (arrowhead) but expansion of anterior expression (arrow).

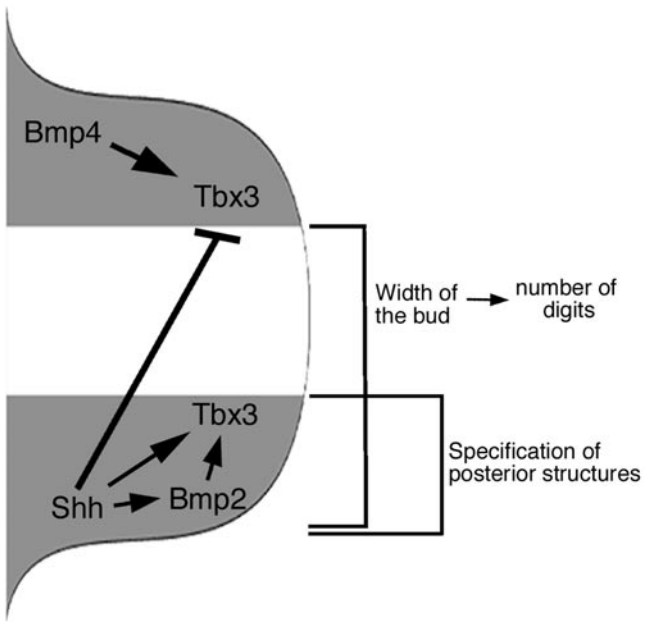

FIG. 6. Diagram outlining how anterior and posterior *Tbx3* expression is regulated. Posterior expression is controlled by the polarising region signalling cascade involving Shh and Bmp2. Anterior expression is controlled by Bmp4 and requires its continuous presence. Moreover, Shh negatively regulates the extent of anterior *Tbx3* expression, and this is related to the width of the bud and number of digits. Posterior *Tbx3* expression could be involved in specifying positional information.

pharyngeal arch development (Garg *et al.*, 2001), although it is not clear whether this is mediated via Bmps.

Our results demonstrate that posterior *Tbx3* expression is regulated by polarising region signalling and therefore could

play a role in specifying position in the limb (Fig. 6). According to fate maps of stage 20 chick wing buds, posterior cells expressing *Tbx3* will give rise to ulna and posterior digits (Vargesson, 1997). This is consistent with the posterior deficiencies in forearm and digits seen in human patients with haploinsufficiency of *Tbx3* (Bamshad *et al.*, 1999; Bamshad, 1997). In contrast, fate maps show that anterior cells which express *Tbx3* in early wing buds contribute rather little to distal structures, and the anterior edge of the middle nonexpressing stripe appears to correspond more or less to the future edge of the hand-plate (Vargesson, 1997). The function of anterior *Tbx3* expression is at present unknown but it is worth noting that the extent of the anterior expression domain is related to the width of the bud which in turn determines the number of digits. Thus, in the case of polydactylous mutants analysed here, the anterior domain of *Tbx3* expression is reduced, while in the $Shh^{-/-}$ embryos, the anterior domain is increased.

ACKNOWLEDGMENTS

We thank Dave Burt and Dave Morrice for *talpid*^{3} and normal quail fertilised eggs; Megan Davey for help with *talpid*^{3} embryos; Malcom Maden (funded by BBSRC) for retinoid-deficient quail embryos; Pascal te Welscher (*Xt/Xt*) and Aimee Zuniga (Shh null) in Rolf Zeller's laboratory for mutant mouse embryos; Mikiko Tanaka for help with RT-PCR and cloning of mouse Tbx3 cDNA; and Natascha Ampunant for help with figures. This work was supported by MRC, The Leverhulme Trust, and BBSRC. M.C.E. is funded by an Anatomical Society studentship.

REFERENCES

Bamshad, M., Le, T., Watkins, W. S., Dixon, M. E., Kramer, B. E., Roeder, A. D., Carey, J. C., Root, S., Schinzel, A., Van Maldergem, L., Gardner, R. J., Lin, R. C., Seidman, C. E., Seidman, J. G., Wallerstein, R., Moran, E., Sutphen, R., Campbell, C. E., and Jorde, L. B. (1999). The spectrum of mutations in TBX3: Genotype/phenotype relationship in ulnar-mammary syndrome. *Am. J. Hum. Genet.* **64,** 1550–1562.

Bamshad, M., Lin, R. C., Law, D. J., Watkins, W. S., Krakowiak, P. A., Moore, M. E., Franceschini, P., Lala, R., Holmes, L. B., Gebuhr, T. C., Schinzel, A., Bruneau, B. G., Seidman, J. G., Seidman, C. E., and Jorde, L. B. (1997). Mutations in human TBX3 alter limb, apocrine, and genital development in ulnar-mammary syndrome. *Nat. Genet.* **16,** 311–316.

Drossopoulou, G., Lewis, K. E., Sanz-Ezquerro, J. J., Nikbakht, N., McMahon, A. P., Hofmann, C., and Tickle, C. (2000). A model for anteroposterior patterning of the vertebrate limb based on sequential long- and short-range Shh signalling and Bmp signalling. *Development* **127,** 1337–1348.

Francis, P. H., Richardson, M. K., Brickell, P. M., and Tickle, C. (1994). Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb. *Development* **120,** 209–218.

Francis-West, P. H., Robertson, K. E., Ede, D. A., Rodriguez, C., Izpisua-Belmonte, J. C., Houston, B., Burt, D. W., Gribbin, C., Brickell, P. M., and Tickle, C. (1995). Expression of genes encoding bone morphogenetic proteins and sonic hedgehog in talpid (ta3) limb buds: Their relationships in the signalling cascade involved in limb patterning. *Dev. Dyn.* **203,** 187–197.

Garg, V., Yamagishi, C., Hu, T., Kathiriya, I. S., Yamagishi, H., and Srivastava, D. (2001). Tbx1, a DiGeorge syndrome candidate gene, is regulated by sonic hedgehog during pharyngeal arch development. *Dev. Biol.* **235,** 62–73.

Gibson-Brown, J. J., Agulnik, S. I., Silver, L. M., Niswander, L., and Papaioannou, V. E. (1998). Involvement of T-box genes Tbx2–Tbx5 in vertebrate limb specification and development. *Development* **125,** 2499–2509.

Grimm, S., and Pflugfelder, G. O. (1996). Control of the gene optomotor-blind in Drosophila wing development by decapentaplegic and wingless. *Science* **271,** 1601–1604.

Gritli-Linde, A., Lewis, P., McMahon, A. P., and Linde, A. (2001). The whereabouts of a morphogen: Direct evidence for short- and graded long-range activity of hedgehog signaling peptides. *Dev. Biol.* **236,** 364–386.

Hamburger, H., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Exp. Morphol.* **88,** 49–92.

He, M., Wen, L., Campbell, C. E., Wu, J. Y., and Rao, Y. (1999). Transcription repression by Xenopus ET and its human ortholog TBX3, a gene involved in ulnar-mammary syndrome. *Proc. Natl. Acad. Sci. USA* **96,** 10212–10217.

Isaac, A., Rodriguez-Esteban, C., Ryan, A., Altabef, M., Tsukui, T., Patel, K., Tickle, C., and Izpisua-Belmonte, J. C. (1998). Tbx genes and limb identity in chick embryo development. *Development* **125,** 1867–1875.

Kopp, A., Muskavitch, M. A., and Duncan, I. (1997). The roles of hedgehog and engrailed in patterning adult abdominal segments of Drosophila. *Development* **124,** 3703–3714.

Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A., and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79,** 993–1003.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H., and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381,** 387–393.

Lewis, K. E., Drossopoulou, G., Paton, I. R., Morrice, D. R., Robertson, K. E., Burt, D. W., Ingham, P. W., and Tickle, C. (1999). Expression of ptc and gli genes in talpid3 suggests bifurcation in Shh pathway. *Development* **126,** 2397–2407.

Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B., and McMahon, A. P. (2001). Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* **105,** 599–612.

Liem, K. F., Jr., Tremml, G., Roelink, H., and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82,** 969–979.

Logan, M., Simon, H. G., and Tabin, C. (1998). Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. *Development* **125,** 2825–2835.

Masuya, H., Sagai, T., Wakana, S., Moriwaki, K., and Shiroishi, T. (1995). A duplicated zone of polarising activity in polydactylous mouse mutants. *Genes Dev.* **9,** 1645–1653.

Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85,** 357–368.

Nieto, A., Patel, K., and Wilkinson, D. G. (1996). In situ hybridisation analysis of chick embryos in whole mount and tissue sections. *Methods Cell Biol.* **51,** 219–235.

Niswander, L., Jeffrey, S., Martin, G., and Tickle, C. (1994). A positive feedback loop coordinates growth and patterning in the limb. *Nature* **371,** 609–612.

Ohuchi, H., Takeuchi, J., Yoshiyasu, H., Ishimaru, Y., Ogura, K., Takahashi, N., Ogura, T., and Noji, S. (1998). Correlation of wing-leg identity in ectopic FGF-induced chimeric limbs with the differential expression of chick Tbx5 and Tbx4. *Development* **125,** 51–60.

Rodriguez-Esteban, C., Tsukui, T., Yonei, S., Magallon, J., Tamura, K., and Izpisua Belmonte, J. C. (1999). The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. *Nature* **398,** 814–818.

Sanz-Ezquerro, J. J., and Tickle, C. (2001). "Fingering" the vertebrate limb. *Differentiation* **69,** 91–99.

Saunders, J. W., Cairns, J. M., and Gasseling, M. T. (1957). The role of the apical ridge of ectoderm in the differentiation of the morphological structure and inductive specificity of limb parts in the chick. *J. Morphol.* **101,** 57–88.

Saunders, J. W., and Gasseling, M. T. (1968). Ectodermal–mesenchymal interactions in the origin of limb symmetry. In "Epithelial–Mesenchymal Interactions" (R. Fleischmajer and R. E. Billingham, Eds.), pp. 78–97. Williams & Wilkins, Baltimore.

Schulte-Merker, S., and Smith, J. C. (1995). Mesoderm formation in response to Brachyury requires FGF signalling. *Curr. Biol.* **5,** 62–67.

Stratford, T., Logan, C., Zile, M., and Maden, M. (1999). Abnormal anteroposterior and dorsoventral patterning of the limb bud in the absence of retinoids. *Mech. Dev.* **81,** 115–125.

Takahashi, M., Tamura, K., Buscher, D., Masuya, H., Yonei-Tamura, S., Matsumoto, K., Naitoh-Matsuo, M., Takeuchi, J., Ogura, K., Shiroishi, T., Ogura, T., and Belmonte, J. C. (1998). The role of Alx-4 in the establishment of anteroposterior polarity during vertebrate limb development. *Development* **125,** 4417–4425.

Takeuchi, J. K., Koshiba-Takeuchi, K., Matsumoto, K., Vogel-Hopker, A., Naitoh-Matsuo, M., Ogura, K., Takahashi, N., Yasuda, K., and Ogura, T. (1999). Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. *Nature* **398,** 810–814.

Tickle, C., Summerbell, D., and Wolpert, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254,** 199–202.

Vargesson, N., Clarke, J. D. W., Vincent, K., Coles, C., Wolpert, L., and Tickle, C. (1997). Cell fate in the chick limb bud and relationship to gene expression. *Development* **124,** 1909–1918.

Wilkinson, D. G., and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225,** 361–373.

Yamada, M., Revelli, J. P., Eichele, G., Barron, M., and Schwartz, R. J. (2000). Expression of chick Tbx-2, Tbx-3, and Tbx-5 genes during early heart development: Evidence for BMP2 induction of Tbx2. *Dev. Biol.* **228,** 95–105.

Yang, Y., Drossopoulou, G., Chuang, P. T., Duprez, D., Marti, E., Bumcrot, D., Vargesson, N., Clarke, J., Niswander, L., McMahon, A., and Tickle, C. (1997). Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb. *Development* **124,** 4393–4404.

Zeng, X., Goetz, J. A., Suber, L. M., Scott, W. J., Jr., Schreiner, C. M., and Robbins, D. J. (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* **411,** 716–720.

Zimmerman, L. B., De Jesus-Escobar, J. M., and Harland, R. M. (1996). The Spemann organiser signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86,** 599–606.

Zuniga, A., Haramis, A. P., McMahon, A. P., and Zeller, R. (1999). Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* **401,** 598–602.

Zuniga, A., and Zeller, R. (1999). Gli3 (Xt) and formin (ld) participate in the positioning of the polarising region and control of posterior limb-bud identity. *Development* **126,** 13–21.

Received for publication March 4, 2002
Revised June 18, 2002
Accepted June 18, 2002
Published online September 12, 2002